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Stability behaviour of antiretroviral drugs and their combinations. 8: Characterization and in-silico toxicity prediction of degradation products of efavirenz

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ABSTRACT

Efavirenz (EFV), an antiretroviral drug, was evaluated for its degradation behaviour in solution state. A total of twelve degradation products were detected on high performance liquid chromatography (HPLC) analyses. Initially, comprehensive mass fragmentation pattern of the drug was established by direct injection and collection of high resolution mass spectrometry (HRMS) and multi-stage tandem mass spectrometry (MS^n) data. Subsequently, LC-HRMS studies were carried on the stability samples containing the degradation products. Eleven degradation products were isolated and subjected to 1D and 2D nuclear magnetic resonance (NMR) studies for their structural confirmation. The collated information was utilized for the characterization of all the degradation products and hence in outlining the comprehensive degradation pathway of the drug. In-silico toxicity of the degradation products was evaluated by TOPKAT analyses.

1. Introduction

Efavirenz (EFV), (4S)-6-chloro –4- (cyclopropylethynyl)-1,4-dihydro-4- (trifluoromethyl)- 2H-3, 1-benzoxazin-2-one is a non-nucleoside reverse transcriptase (NNRT) inhibitor of human immunodeficiency virus type 1 (HIV-1) [1]. Literature review revealed that comprehensive report on stability behaviour of EFV is not available in the literature. A publication exists that involved characterization of two alkaline degradation products of the drug, viz., an amino alcohol and a quinoline derivative [2]. Multiple other studies also investigated susceptibility of the drug under variety of conditions, i.e., hydrolytic, oxidative and photolytic, while attempting to develop stability-indicating analytical methods [3–11]. These investigations reported variable extent of drug degradation, and also lacked characterization of the degradation products.

Therefore, during our current series of stability studies on antiretroviral drugs and their combinations [12–18], we also targeted to explore comprehensive degradation behaviour of EFV. The drug was found to be labile under acidic and basic stress conditions. HPLC analyses of the same revealed formation of twelve degradation products in total. All of them were subjected to liquid chromatography- high resolution mass spectrometry (LC-HRMS) for their characterization. Among them eleven degradation products, which could be isolated, were subjected to 1D and 2D nuclear magnetic resonance (NMR) for their structure confirmation. The results and observations helped to delineate degradation pathway of the drug under the investigated stress conditions. Additionally, in-silico toxicity of all the degradation products was evaluated by using TOPKAT software.

2. Experimental

2.1. Chemicals and reagents

Pure EFV was gifted by M/S Aurobindo Pharma Ltd. (Hyderabad, India). Hydrochloric acid (HCl) and sodium hydroxide (NaOH) of analytical grade were purchased from LOBA Chemie Pvt. Ltd. (Mumbai, India) and Ranbaxy Laboratories (S.A.S. Nagar, India), respectively. HPLC grade methanol (CH_3OH) was purchased from Aldrich (St. Louis, MO, USA). Deuterated water (D_2O, 99.9%), deuter-

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ated methanol (CD_3OD, 99.8%) and deuterated acetonitrile (CD_3CN, 99.8%) were also purchased from Aldrich. Buffer salts and all other chemicals were of analytical reagent grade. Ultra pure water (H_2O) was obtained from ELGA water purification unit (Bucks, England).

2.2. Apparatus and equipments

The photostability chamber was equipped with an illumination bank on the inside top, which consisted of a combination of three UV (OSRAM L18 W/73) and three white fluorescent (Philips, Trulite) lamps. Lux meter (model ELM 201, Escorp, New Delhi, India) and a near UV radiometer (model 206, PRC Krochmann GmbH, Berlin, Germany) were used to measure visible illumination and near UV energy, respectively.

HPLC studies were carried out on a LC-2010C HT liquid chromatograph (Shimadzu, Kyoto, Japan), which was equipped with a SPD-M20A prominence diode array detector. Pursuit XRs C18 (5 μ, 250 × 4.6 mm, Varian Inc., Lake Forest, CA, USA) LC column was used for analytical studies. Pursuit XRs C8 (5 μ, 250 × 4.6 mm, Varian Inc.), Luna C18 (10 μ, 250 × 30.00 mm, Phenomenex, Torrance, CA, USA) and Kingsorb C18 (5 μ, 250 × 10.0 mm, Phenomenex) columns were used for isolation of the degradation products.

Direct HRMS study on the drug was conducted on Maxis spectrometer (Bruker Daltonics, Bremen, Germany), while LC-HRMS studies were carried out using LC-ESI-Q-TOF-MS, in which the LC part consisted of 1100 series HPLC (Agilent Technologies, Waldbronn, Germany) comprising of an on-line degasser (G1379A), binary pump (G1312A), auto injector (G1313A), column oven (G1316A) and the PDA detector (G1315B). The HRMS system consisted of a MicrOTOF-Q spectrometer (Bruker Daltonics). System control and data acquisition were done by Hystar software (version 3.1) from the same source. The calibration solution used was a 5 mM sodium formate. LTQ-XL-MS 2.5.0 (Thermo, San Jose, CA, USA) mass spectrometer was used for tandem mass experiments. The mass spectra were acquired and processed using Xcalibur software (version 2.0.7 SP1). NMR studies were performed on a FT-NMR AV-400 (Bruker BioSpin International AG, Zug, Switzerland) and JNM-ECA 500 MHz NMR spectrometer (JEOL, Tokyo, Japan). The data of the two machines were processed by TopSpin 3.5pl5 and Delta v5.0.4.4 software, respectively.

pH/Ion analyzer model PB-11 from Sartorius (Göttingen, Germany) was used to check pH of all the solutions. Other equipments used were sonicator (3210, Branson Ultrasonics Corporation, Danbury, CT, USA), precision analytical balance (AG 135, Mettler Toledo, Schwerzenbach, Switzerland) and auto pipettes (Eppendorf, Hamburg, Germany).

2.3. Stress studies

The drug was subjected to hydrolytic, oxidative and photolytic stress studies according to the protocol described in our previous publications [19,20]. Drug stock solutions (4 mg/ml) were prepared in CH_3CN:H_2O (60:40 v/v). Final drug concentration in all stress solutions was 2 mg/ml. Hydrolytic studies were carried out in CH_3CN:H_2O (60:40 v/v, neutral), 2 N HCl and 0.02 N NaOH solutions. The temperature and time of hydrolytic reactions were 80 °C and 2 h, respectively. Oxidative stress studies were carried out in 15% hydrogen peroxide (H_2O_2) and 100 mM% azobisisobutyronitrile (AIBN, 200 mM% solution in CH_3CN:CH_3OH (80:20 v/v)) for 48 h. Photolytic studies were conducted on drug solutions prepared by diluting drug stock by two times with the same solvent. The resultant solution was exposed for 14 days in a photostability chamber equipped with the lamps yielding intensity of 3500 lx cool white and 0.7 W/m^2 UV-A radiations. Dark control was kept in parallel for comparison. Samples were diluted two times with CH_3CN:H_2O (60:40 v/v) before injecting into HPLC. By employing a method involving the mobile phase composed of methanol (A) and 10 mM ammonium formate (pH 3.75) (B) in a gradient mode (Tmin/%B; T0/40; T5/40; T25/10; T33/10; T38/40; T45/40). The column oven temperature was 30 °C. The detection wavelength, flow rate and injection volume were 254 nm, 1 ml/min and 10 μl, respectively.

2.4. HRMS, MS^n and LC-HRMS studies

A 10 μg/ml solution was directly injected into the HRMS system for establishment of the fragmentation pattern of the drug. MS^n studies were similarly conducted by directly injecting 10 μg/ml solution of the drug into the LTQ-XL system at a flow rate of 10 μl/min. The HRMS and MS^n instrument parameters were to obtain suitably for good intensity of molecular and fragment ions (Table S1). For LC-HRMS, the LC method used was similar to the HPLC method employed for analyses of the stressed samples. However, to prevent condensation in the ionization source, the solvent flow into the MS system was reduced from 1 ml/min to 200 μl/min, using a diversion valve. Moreover, in case of LC-HRMS, instrument parameters were modified suitably to increase response of the degradation products (Table S1).

2.5. NMR studies on the drug and degradation products

Initially, the drug was subjected to 1D and 2D NMR studies in CD_3CN for later comparison with NMR data of the degradation products. Eleven out of twelve degradation products were enriched by optimizing the stress parameters (stressor strength, temperature and time) in which they were formed in relatively higher concentration. They were isolated on semi-preparative and/or analytical HPLC columns, employing different HPLC methods for different conditions. The mobile phase used for isolation contained methanol and acidified water. In each case, the collected HPLC fraction was dried on a rotary evaporator and the residue was subjected to 1D and 2D NMR studies after preparing solution in CD_3CN and/or CD_3OD. D_2O was used for hydrogen/deuterium (H/D) exchange studies.

2.6. In-silico toxicity prediction

TOPKAT (TOxicity Prediction by Komputer Assisted Technology, Discovery Studio 2.5, Accelrys, Inc., San Diego, CA, USA) was employed to predict potential toxicity of the degradation products [21]. Probability values from 0.0 to 0.30 are considered as low probabilities for any toxicological end point, whereas those greater than 0.70 are considered as high probabilities [22]. The values from 0.3 to 0.7 fall in an in-determinate zone.

3. Results and discussion

3.1. HPLC analyses of the stressed samples

A total of twelve degradation products were observed under acidic and basic solution state stress conditions (Fig. 1). Acidic stress (2 N HCl) resulted in the formation of all twelve degradation products, while only two (E8 and E12) were generated in the basic (0.02 N NaOH) environment. The drug did not show any degradation under oxidative and photolytic conditions.

3.2. Mass studies on the drug

The HRMS line spectrum of the EFV under ESI +ve mode is shown in Fig. S1. Table 1 lists data acquired from HRMS and MS^n studies. The best possible molecular formulae and exact masses for the fragments were calculated using an elemental composition calculator.